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Fate of ^{3}H-thymidine Labelled Myogenic Cells in Regeneration of Muscle Isografts

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Summary. Intact and denervated extensor digitorum longus (EDL) muscles of 20-day-old inbred Lewis-Wistar rats were labelled with ^{3}H-thymidine. Ninety minutes after the injection of the isotope 4.0% of the nuclei were labelled in the intact (i.e. innervated) and 9.6% in the muscles, denervated 3 days before administration of the isotope. The labelled EDL muscles were grafted into the bed of the previously removed EDL muscles of inbred animals and these isografts were studied 30 days later.

In the EDL muscles, regenerated from innervated isografts only occasionally labelled endothelial cells were found whereas in the muscles regenerated from denervated isografts also parenchymal muscle nuclei were regularly labelled. The incidence of labelled nuclei in the regenerated EDL muscles was, however, about 20 times lower than in the donor EDL muscles. The present experiments provide a direct proof of utilization of donor satellite cell nuclei for regeneration in grafted muscle tissue. With respect to the low incidence of labelled nuclei in regenerated EDL muscles, other sources of cells apparently also contribute to the regeneration process.

Key words: Skeletal muscle – Transplantation – Muscle regeneration – Historadiography.

Introduction

The description of regeneration of muscle fibres from grafted minced muscle tissue (Studitsky, 1959) has initiated great interest in studies on mechanisms of muscle regeneration. However, the origin of myogenic cells in the regenerating muscle has not yet finally been solved (Carlson, 1973). It is generally assumed that myogenic cells of the grafted muscle tissue are the only source of the regenerating muscle. Basically, two concepts concerning the myogenic origin of regenerating tissue prevail. The regeneration of muscle fibers is ascribed either to the activation of satellite cells, i.e. mononuclear cells located between

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a muscle fiber proper and its basement membrane (Mauro, 1961), or to myonuclei "broken off" from the damaged or degenerated muscle fibers (Reznik, 1970). Morphological observations of transition between satellite cells and myoblasts have been described in regeneration (Shafiq *et al.*, 1967) and there is also good evidence for transformation of satellite cells into myoblasts following injury (Church, 1970). In single and double injury experiments, myoblasts, recognized as ^{3}H-thymidine labelled nuclei, were however, observed to be incorporated into regenerating muscle fibers (Walker, 1972). These observations were interpreted in the sense that nuclei of regenerating muscle fibers are derived from pre-existing myonuclei (Walker, 1972). The source of the regenerating nuclei may, however, not be only a myogenic one. Muscle fibers might also regenerate from undifferentiated mesenchymal cells of loose connective tissue (Levander, 1964; Polezhaev, 1973) inside or outside the graft or from mesenchymal cells of the remaining tendon, inducing their transformation. This possibility is also suggested by regeneration of muscle tissue observed after grafting of liver cells into the bed of a previous removed muscle (Gutmann *et al.*, 1976).

In the present experiments the fate of ^{3}H-thymidine labelled myogenic nuclei of muscles grafted into the bed of previously removed muscles of inbred young rats, i.e. isografts was investigated. According to Moss and Leblond (1971) in 17-day-old rats all nuclei labelled 90 minutes after injection of ^{3}H-thymidine are those of satellite cells. These cells would therefore be mainly expected to be incorporated into the regenerating muscle fibers of the isografts in our experiments. The number of dividing and/or labelled satellite cells, is, however, low under normal conditions and decreases progressively with age. These cells, which may represent a reserve population of "presumptive" myoblasts and other cell types increase in number under different pathophysiological conditions such as, for instance, denervation (Gutmann and Young, 1944; Lee, 1965; Hess and Rossner, 1970), injury (Teravainen, 1970) or compensatory hypertrophy (Aloisi *et al.*, 1973; Hanzlíková *et al.*, 1975). Therefore, isografts of previously denervated muscles were also used in one set of our experiments.

Materials and Methods

Animals and Isotope. The experiments were performed in 10 twenty-day-old Lewis-Wistar, inbred male rats (60 generations) with a body weight of about 30 g. 6-^{3}H-thymidine (specific activity 19 Ci/mmol) produced by ÚVVVR, Prague was used.

Experimental Methods. 3 intact (i.e. with innervated limbs) animals and 3 animals in which bilateral section of the peroneal nerve was carried out three days before, received intraperitoneally 5 μCi of ^{3}H-thymidine per g/b weight and were sacrificed 90 minutes after the injection for histological and autoradiographic analysis of the "donor" muscle while the muscle of the other leg was orthotopically grafted into the bed of the previously removed extensor digitorum longus (EDL) muscle of the intact inbred recipient. For a detailed description of the "free graft"-transplantation technique see Carlson and Gutmann (1974). Four weeks later the regenerated EDL muscles were removed for autoradiographic analysis.

Histology and Autoradiography. The muscles were fixed in 10% formaline+0.5% TCA for 24 hours. After washing in tap water and dehydration in alcohols they were embedded in Paraplast.

Autoradiograms were prepared from the 5 μ thick cross sections by Stripping film Kodak AR 10. The autoradiograms were exposed at +4° C for a period of 22 or 48 days. After developing in Kodak 19b solution (4 min) they were fixed and washed and stained with Mayer's hematoxylin and eosin.

Evaluation of Autoradiograms. Autoradiograms were evaluated at a magnification of 1,000. Background ranged from about 0.3 to 1 grain per 100 μ^2 under the shorter and longer exposition time respectively. Five sections were evaluated in each muscle and set of exposition.

For determination of the labelling Index (L.I.) about 4,000 nuclei were counted in each muscle. Due to the low number of animals in the individual experimental groups and the primary qualitative aims of the experiments, arithmetic means and the values measured in individual animals only are reported. L.I values refer to 48 days exposed autoradiograms.

Results

a) *General Observations.* Morphological and functional recovery of the EDL muscle could be observed in all animals in which transplantation of the muscle isografts was carried out. This follows from the normal macroscopical appearance of the regenerated EDL muscles at the time of sacrificing the animals (30 days after transplantation) and from the recovery of the spreading reflex of the toes, effected by the EDL muscle (Gutmann, 1942). When comparing these criteria in animals which received either innervated or denervated isografts, the degree of recovery was found to be higher after transplanting denervated than innervated EDL muscles.

b) ^{3}H-thymidine Incorporation in Donor EDL Muscles. Both in the intact (i.e. innervated) and the denervated EDL muscles labelled nuclei were regularly observed. In spite of the low resolving power of the light-microscope autoradiography it was evident that most of them were of parenchymal type, resembling mainly satellite-like cells by their cup-like localisation on the muscle fiber (Figs. 1, 2). Only a small part of labelled nuclei belonged to the endothelial cell group or to other mesenchymal cell types. The number of grains per nucleus ranged from about 15 and 30 to 60 and 90 in autoradiograms exposed for 22 and 48 days respectively. Some nuclei, especially after longer exposition, were even diffusely black so that grain counting was not possible. When estimating the incidence of labelled cells all nuclei were counted, avoiding in this way difficulties of their definition, caused by low resolving power of light microscopy. The mean labelling index (L.I.), expressed as a percentage of the total number of nuclei, amounted on the average to 4.0% in the *innervated* donor EDL muscles. The values measured in individual animals were 1.3%, 4.9% and 5.8% respectively. In the animals in which the EDL muscles were *denervated* 3 days before the isotope injection, the incidence of labelled nuclei was considerably higher. The L.I. was on the average 9.6%. In the individual animals 4.8, 7.1 and 16.9 per cent of nuclei respectively were found to be labelled.

c) Labelling of the Regenerated EDL Muscles Originating in Transplanted ^{3}H-thymidine Labelled Isografts. In the EDL muscles regenerated from the *innervated* isografts no labelled nuclei of muscle type could be detected. Only exceptionally

a labelled endothelial-like cell nucleus could be observed in a muscle of one animal of this experimental group. In the EDL muscles regenerated from the *denervated* isografts 2 to 5 labelled nuclei (corresponding to about 0.2 to 0.5% of the total number of nuclei in the section) were regularly observed. The labelled nuclei belonged both to the muscle parenchymal type (Figs. 4–6) and occasionally also to mesenchymal-like cells (Fig. 3). The number of grains per nucleus was relatively high. It ranged from about 15 to 50 grains after shorter exposition time and about 20 to 80 in autoradiograms exposed for a longer time.

Discussion

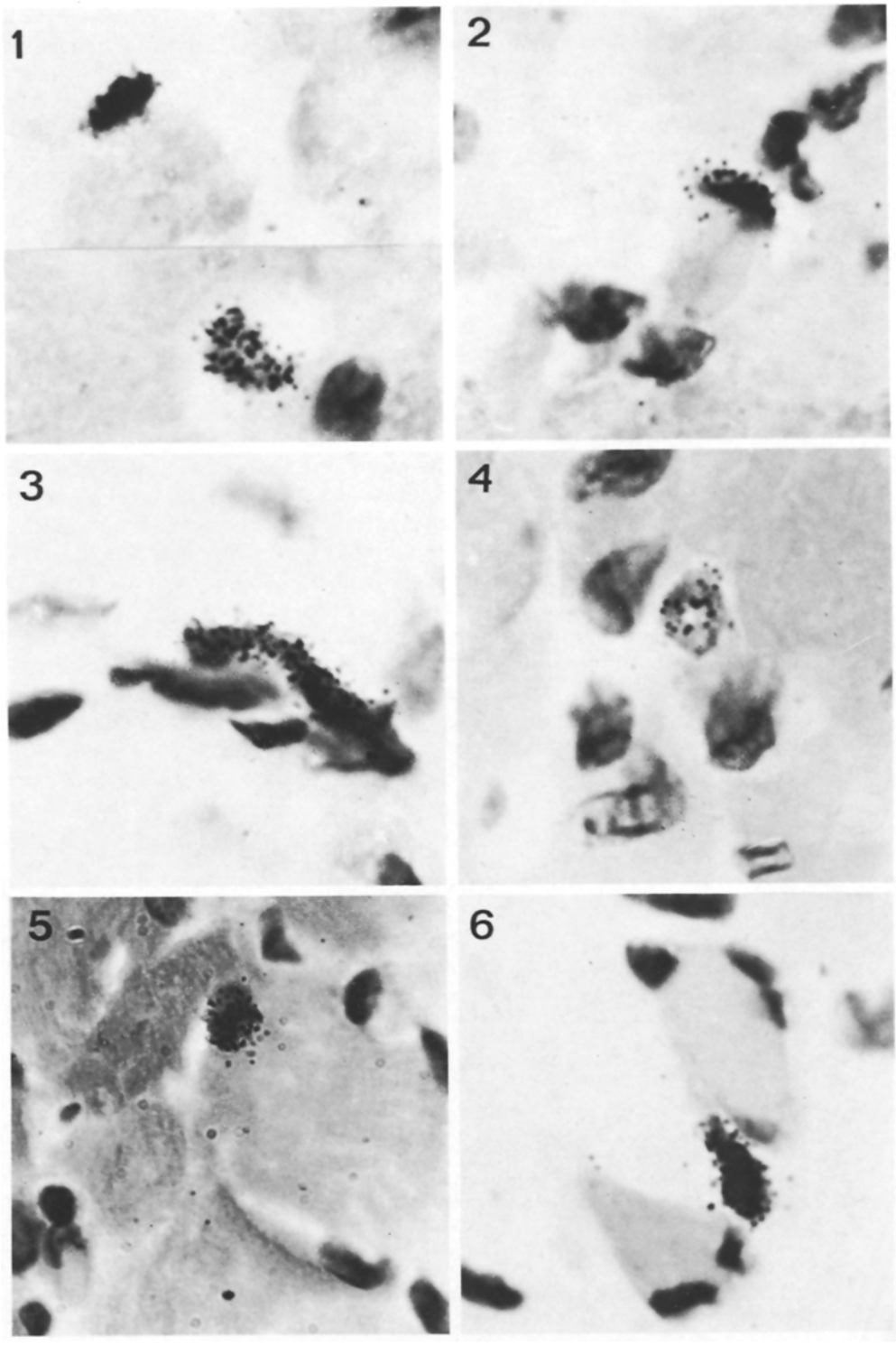
The occurrence of labelled nuclei in regenerated EDL muscles presents direct evidence for the assumption that some nuclei of the grafted muscle are integrated into the regenerating muscle tissue. Such a proof appeared to be necessary with respect to the massive degeneration of the grafted tissue occurring in the first days after transplantation. The relatively high grain counts in the nuclei integrated in regenerating muscles as well as the relatively small scatter of grain densities provide evidence against the possibility that the labelling is only due to reutilization of break-down products of donor DNA.

Exact cytological definition of the labelled donor cells integrated into the regenerating muscle is in our experiments hampered by the low resolving power of light microscopy, which, however, allows easy detection and representative quantification of the labelling. Previous experiments (Moss and Leblond, 1971) on an ultrastructural basis have shown that the nuclei, labelled in muscles of 17-day-old rats, 90 minutes after the ^{3}H-thymidine injection, were exclusively those of satellite type. We may therefore reasonably assume that also in our experiments, carried out under similar conditions, the labelled nuclei of the isografted muscles were mostly those of satellite cells and that the labelled nuclei observed in regenerated muscles originated from the population of satellite cells surviving in the isograft. This origin of labelled nuclei in regenerated muscles is supported by the satellite-like appearance and position of labelled nuclei in donor EDL muscles. Labelled nuclei 30 days after transplantation appeared, however, only in muscles regenerating from previously denervated grafts where the population of satellite (Hess and Rossner, 1970) and other cells (Gutmann and Young, 1944) is known to increase in number due to cell division (Blunt *et al.*, 1975). Consistently with this a higher number of

Figs. 1 and 2. Labelled nuclei of the 3-day-denervated EDL muscle 90 minutes after injection of ^{3}H-thymidine. Autoradiogram, exposition 22 days. Hematoxylin and eosin. ×900

Fig. 3. Labelled mesenchymal cell nuclei (adjacent to the wall of a blood vessel) in regenerated EDL muscle after transplantating denervated, DNA-prelabelled isograft. Autoradiogram, exposition 22 days. Hematoxylin and eosin. ×900

Figs. 4–6. Labelled nuclei of regenerated muscle fibers of the EDL muscle after transplanting denervated, DNA-prelabelled muscle isograft. Autoradiogram, exposition 22 days. Hematoxylin and eosin. ×900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labelled cells appeared in denervated donor EDL muscles in the present experiments and labelled nuclei were found in regenerated EDL muscles in this set of transplantations. It is interesting that a better, functional recovery of regenerates, with respect to contractile properties occurred also after grafting denervated muscle (Vyskočil *et al.* 1973).

The number of integrated labelled nuclei in the regenerated muscle is, however, very low. It represents only about 5% of the initially labelled donor cells. Taking into consideration this fact, the absence of labelled cells in the EDL regenerating from innervated grafts should not be necessarily considered as a qualitative difference in utilization between intact and denervated grafted muscles but might be attributed to the "subthreshold" number of satellite cells surviving transplantation.

Thus, it appears to be established that labelled satellite cells can be incorporated in regenerating isograft. The further fate of incorporated labelled satellite cells in the regenerated EDL muscle remains, however, unclear and needs further study. The majority of the nuclei in the regenerated EDL muscles remained unlabelled. Underestimation of the number of integrated labelled nuclei due to dilution of radioactivity by cell division is improbable because of the relative high grain densities and their low scatter in the labelled population of nuclei. This finding suggests an origin of muscle cells from other than satellite type of cells, as for instance from myonuclei (Walker, 1972) or non-muscle cells, present inside or outside the graft (Levander, 1964; Polezhaev, 1970; Gutmann *et al.*, 1975). It is generally assumed, that cells redifferentiate only into their former cell types, exhibiting stability of determination. However, the possibility of a redifferentiation into different cell types, exhibiting metaplasia, is also suggested by studies on cell differentiation during limb regeneration (Hay, 1962; Steen, 1970).

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